



Temporal stability of *Orbicella annularis* symbioses: a case study in The Bahamas

¹College of Life and Environmental Sciences,
University of Exeter, Stocker
Road, Exeter EX4 4QD, UK.

²Global Change Institute,
University of Queensland, St
Lucia, QLD 4072, Australia.

³Wageningen Marine Research,
Wageningen University and
Research, P.O. Box 77, 4400 AB
Yerseke, The Netherlands.

⁴School of Biological and Marine
Sciences, Plymouth University,
Drake Circus, Plymouth PL4
8AA, UK.

⁵Marine Spatial Ecology Lab,
School of Biological Sciences,
University of Queensland, St
Lucia, QLD 4072, Australia.

* Corresponding author email:
<e.kennedy1@uq.edu.au>.

Emma V Kennedy^{1, 2, *}

Linda Tonk^{2, 3}

Nicola L Foster⁴

Peter J Mumby⁵

Jamie R Stevens¹

ABSTRACT.—*Orbicella annularis* (Ellis and Solander, 1786), a key reef building species, is unusual among Caribbean corals in the flexibility it displays in its symbioses with dinoflagellates in the family Symbiodiniaceae. This variability has been documented at a range of spatial scales; from within and between colonies to scales spanning the entire species range. However, temporal variability in Symbiodiniaceae communities found within *O. annularis* colonies is not well understood. Evidence suggests that symbiont communities in this coral species fluctuate temporally in response to environmental stressors (sporadic changes in abundance and in community composition). In this study, we investigated temporal stability of symbiont communities in *O. annularis* at four sites in The Bahamas over a period spanning 6 yrs. While the dominant symbiont species, *Breviolum minutum* (LaJeunesse et al.) J.E.Parkinson & LaJeunesse (formerly ITS2-type B1), remained stable across four patch-reef study sites, finer resolution molecular techniques revealed inter-annual variability in the presence/absence of cryptic species *Durisdinium trenchii* (LaJeunesse) LaJeunesse (formerly ITS2-type D1a). *Durisdinium trenchii* is known to play a role in resistance to environmental stress and may have a protective effect under warm conditions. These results suggest that, while it might take an extreme environmental perturbation to trigger a long-term shift in the dominant symbiont, at background levels, less prevalent symbiont taxa are likely to be continually shuffling their relative abundances as they change in response to seasonal or environmental changes.

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Orbicella annularis (Ellis and Solander, 1786) is atypical among Caribbean corals in displaying spatial and temporal variability in the dinoflagellate symbiont types with which it partners (Thornhill et al. 2006b). Importantly, with Symbiodiniaceae conferring different physiological properties, this could have ecological consequences for these reef building organisms; for example, regarding bleaching responses

(Kennedy et al. 2016). Over short timescales (days to weeks), the relative proportions of clades B, C, A, and D (recently recognized to be genera *Breviolum*, *Cladocopium*, *Symbiodinium*, and *Durisdinium* by LaJeunesse et al. 2018) have been shown to experience appreciable shifts in response to natural (Rowan et al. 1997, LaJeunesse et al. 2009) and experimentally-induced (Toller et al. 2001) bleaching events. Recovering corals may also exhibit “successional” shifts in symbiont populations over timeframes of months (Toller et al. 2001, LaJeunesse et al. 2009) to years (Thornhill et al. 2006b). Competitive interactions among symbiont taxa may also occur over longer timeframes (i.e., years to decades) within colonies as coral growth causes slow changes in microenvironments (such as corallites shifting from the tops to the sides of ramets; Toller et al. 2001b). This has been demonstrated by experimental manipulations of irradiance gradients in *O. annularis*, producing changes in the distribution of *Breviolum* and *Cladocopium* species within a colony in the space of 6 mo (Rowan et al. 1997).

Nonetheless, while substantial field and laboratory evidence exists for varying degrees of symbiont “shuffling” in *O. annularis* in response to mild to severe environmental change, a relatively high degree of temporal fidelity in symbiont partnerships is reported in the majority of individual hosts. *Orbicella annularis* has been compared to Caribbean brooding corals [e.g., *Agaricia agaricites* (Linnaeus, 1758), *Porites astreoides* Lamarck, 1816, and *Siderastrea radians* (Pallas, 1766)] in terms of the substantial stability in symbiont communities it experiences over longer time periods (Thornhill et al. 2006a). Rowan et al. (1997) reported temporal stability of clade type in their field control *O. annularis* colonies over periods of 6 mo. Toller et al. (2001)’s experimental manipulations revealed that recovering *O. annularis* generally showed identical symbiont clades as they did prior to treatment, except in the cases of severe depletion (caused by disease or temperature-induced bleaching) where new clades would become established. Thornhill et al. (2006b) went on to demonstrate that even in severely bleached colonies, symbiont communities tended to slowly revert to their original symbioses, with up to 5 yrs required for complete post-bleaching reversion. On two reefs, tagged *O. annularis* colonies showed gradual changes in both the identity and proportion of ITS2 symbiont types D1a, C3, and B1 at one reef, and D1a, B1, and B10 in another until 2002, when a stable dominance of B1 or B10 was eventually re-established (Thornhill et al. 2006b). Another study, which examined population genetic variation in *Breviolum* populations hosted by *O. annularis* at two sites in The Bahamas, reported a high degree of reef endemism at the *Breviolum minutum* (LaJeunesse et al.) J.E.Parkinson & LaJeunesse (formerly B1) population level, with little evidence of temporal change in endosymbiont type during a 2-yr sampling period (Thornhill et al. 2009).

The aim of the present study was to assess temporal stability of *O. annularis* symbiont communities at four sites within the Bahamian archipelago. *Orbicella annularis* from The Bahamas (Thornhill et al. 2009, Kennedy et al. 2016) are known to associate with *B. minutum* (until recently more commonly referred to as *Symbiodinium* B1; LaJeunesse et al. 2018). Symbiont communities inhabiting *O. annularis* colonies at four sampling locations adjacent to two Bahamian islands were sampled four times between 2006 and 2012. During this period, several well-documented thermal stress events occurred, which may have disrupted the balance of symbionts.

Specifically, we investigated how the relative proportions of symbionts hosted by *O. annularis* at Propeller and School House reefs (New Providence Island) and

Seahorse and Snapshot reefs (San Salvador Island) varied across a 6-yr sampling period. Secondly, we explored how detectable background occurrences of thermally-tolerant *Durussdinium trenchii* (LaJeunesse) (referred to in previous literature as D1a) hosted by *O. annularis* at these sites varied with time. From this, we explore whether the observed partitioning of Symbiodiniaceae diversity represents a snapshot in time of a dynamic partnership, or whether it represents a suite of more stable symbioses; dependent on this, conclusions regarding symbiont traits can be derived, and better-informed decisions regarding placement of marine reserves and other conservation actions can be formulated (Mumby et al. 2011). The consequences of symbiont shuffling are important for understanding the capacity of corals to respond to thermal stress events.

MATERIALS AND METHODS

SAMPLE COLLECTION.—Field sites, established as part of a study on *O. annularis* (Foster 2007), were accessed using scuba during the third week in June in 2006, and again at the exact same time of year in June 2010, June 2011, and June 2012. Propeller and School House reefs are located approximately 6 km apart on the southwest of New Providence Island (Fig. 1). Propeller reef (25.0064°N, 77.5524°W) is located approximately 1 km from a power station waste-water outflow pipe, and was dominated by large colonies of *O. annularis*. School House reef (24.9734°N, 77.5051°W), a similar (but slightly smaller) patch reef east of Propeller reef, lies farther (approximately 3.5 km) offshore.

Snapshot and Seahorse reefs are located on San Salvador Island, lying southeast of New Providence on the eastern limit of The Bahamas archipelago, and more exposed to the open waters of the Atlantic Ocean. Seahorse reef (24.1582°N, 74.4839°W), an extensive patch reef with a number of large gorgonians and several *Acropora* spp. across the site, is located 4.5 km off the most northerly tip of San Salvador. Snapshot reef (24.0314°N, 74.5297°W), located on the western leeward side of San Salvador, is slightly more sheltered. Again, the site was dominated by *O. annularis*. All sites were between 3 and 4 m deep.

A 10-m wide circular sampling plot encompassing multiple *O. annularis* colonies was established at each location sensu Foster (Foster 2007). *Orbicella annularis* typical growth form is multiple disjunct columns (or “ramets”), often with senescent margins, emerging from a basal colony (Weil and Knowlton 1994). Each year, individual ramets were randomly sampled from every *O. annularis* colony within each plot (to a total of 30 samples per plot), with the target number of samples collected across the four sites and 4 yrs being 480. Each spatially independent ramet (i.e., with no connected tissue) was sampled only once. Seahorse Reef was not accessible in 2010, so the total number of collected samples was 450. The location of each sampled ramet was mapped by recording distance (to the nearest 5 cm) from the center of the sampling plot (marked with a fixed stake), and bearing (°). Individual ramets were not tagged, which means sampling between years did not target the exact same ramet through time. Molecular analysis of 2006 samples revealed ramets within each sampling plot were genetically diverse. While up to three clonemates were detected within a plot (e.g., Seahorse Reef had three sets of clones; independent colonies with identical genotypes), host genetic variability was proven to have little to no influence on symbiont species hosted (see Kennedy et al 2016 for details). Bahamian *O.*

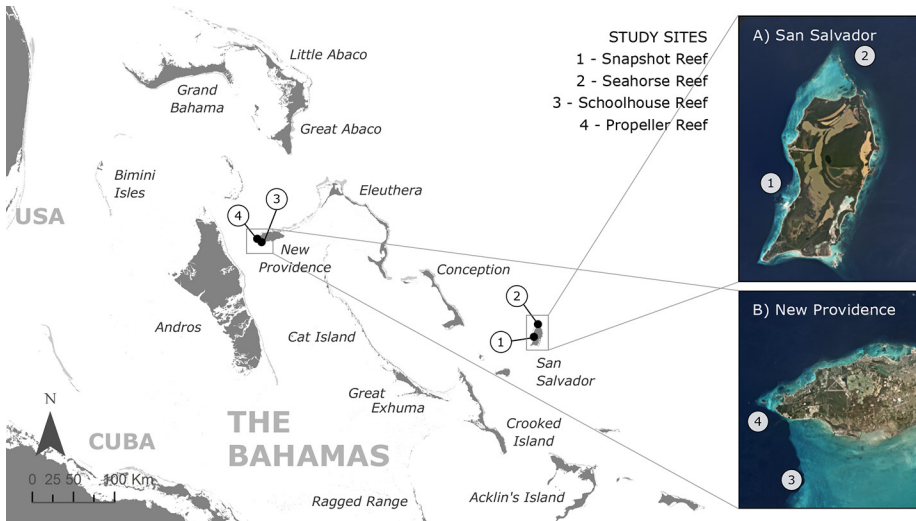


Figure 1. Map of the Bahamian archipelago, showing study site locations. Inset (A) San Salvador Island, (B) New Providence Island.

annularis hosts displayed a significant amount of diversity compared to other Wider Caribbean sites sampled (Kennedy et al 2016). An approximately 1-cm² sample was chipped from the edge of each ramet using a hammer and a small chisel. Samples were collected in separate labelled plastic ziplock bags, and on returning to the shore were preserved in 90% ethanol and stored at 4 °C. GPS coordinates, time, weather conditions, and reef position of each site were also recorded.

MOLECULAR ANALYSES.—Holobiont tissue was homogenized and both host and symbiont DNA extracted overnight using Qiagen DNEasy Blood and Tissue kits. Roughly 1 cm² of tissue was removed from the skeleton using a scalpel, and overnight lysis performed as per the manufacturer's instructions. Extracted DNA was amplified in a PCR reaction using *Symbiodinium*-specific rDNA primers "ITS2 Clamp" [modified "ITS-reverse" primer with an additional 39 bp GC clamp (underlined), 5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GGG ATC CAT ATG CTT AAG TTC AGC GGG T-3'] and "ITSintfor2" (5'-GAA TTG CAG AAC TCC GTG-3') using a PCR protocol [95 °C × 5 min; followed by 30 cycles of 94 °C (45 s), 57 °C (45 s), 72 °C (60 s); with a final annealing step of 59 °C for 20 min], based on LaJeunesse (2002). The reaction mix comprised 1× PCR reaction buffer, 2.5 mM MgCl₂, 0.2 mM dNTPs, 2 U Taq DNA Polymerase, and 0.6 μM primer in a 12.5 μl reaction. One microliter of the final eluate was used as a template for amplification. DNA quality in the final eluate was assessed using a nanodrop.

Denaturing gel gradient electrophoresis (DGGE) was used to identify bands representing Symbiodiniaceae species within the PCR product for each individual (following Kennedy et al. 2016). PCR products were electrophoresed on a polyacrylamide denaturing gradient gel for 14 h at 114 V (INGENYphorU-2×2 system, Ingeny). To prepare the gel, a 0% [20 ml of 40% acrylamide (37.5:1 acrylamide:bis-acrylamide), 2 ml of 50× TAE, and 78 ml dH₂O] and a 100% [20 ml of 40% acrylamide, 40 ml formamide, 42 g urea, and 2 ml of 50× TAE] denaturant acrylamide solution were prepared beforehand, and mixed to get a 60% (12.8 ml 0% solution: 19.2 ml 100%

solution) and a 40% solution (12.8 ml 100% solution: 19.2 ml 0% solution). To each solution, 120 μ l ammonium persulphate (APS) solution (0.1 g APS: 1 ml dH₂O), and 18 μ l TEMED (Bio-Rad) were added. Gels (28 \times 18 cm) were cast using a manual gradient mixer, and then left to set for 2 hrs. PCR product (15 μ l) from every individual was loaded into DGGE wells after mixing with 5 μ l bromophenol blue loading buffer (15% Ficoll, 0.25% xylene cyanol FF, 0.25% bromophenol blue). Gels were run overnight for 14 hrs at 114 V, at a constant temperature of 60 °C. An ITS2 standard (provided by the Hoegh-Guldberg lab, UQ) was run in the first lane of each gel. After 14 hrs, the gel was gently transferred to a staining bath and covered with SybrGreen I nucleic acid gel stain from Invitrogen (stock was diluted 1:10,000 in 1 M TAE buffer).

Imaged gels were examined carefully by eye and scored for types, with comparison to a database of other gels used to help identify haplotypes. Representatives of every discrete, prominent band were excised under a UV-transilluminator using 10 μ l tips and stored at 4 °C overnight in 1.5 ml eppendorf tubes containing 30 μ l RNase free water. Reamplification was performed with 1 μ l eluate, using ITSinfor2 and ITS2-reverse lacking the GC-clamp. Two microliters of the PCR product was then cleaned using Exo-sap (per 100 samples: 5 μ l Exonuclease 1 (20U μ l⁻¹), 10 μ l Exonuclease buffer, and 85 μ l dH₂O, along with 20 μ l Antarctic phosphatase (5U μ l⁻¹), 10 μ l buffer and 70 μ l dH₂O). Two microliters was added to each sample and put in the thermocycler at 37 °C for 15 min and a further 15 min at 80 °C. Following the determination of concentration of each sample on the nanodrop, samples were diluted to a suitable concentration (6–12 ng μ l⁻¹) for sequencing. The product was sequenced in both directions using both forward and reverse amplification primers separately (Macrogen). A sequence alignment was performed in Clustal X and checked by eye, prior to comparison against a database of all known Caribbean *Symbiodinium* types in GeoSymbio database (Franklin et al. 2012).

Real-time PCR in conjunction with high resolution melt (HRM) analysis were used to screen each *O. annularis* ramet specifically for the presence or absence of low abundance *Durusdinium* (following Kennedy et al. 2015a). A 312 base pair target region specific to *Symbiodinium* clade D, located in domain 2 of the LSU gene, was amplified using published qPCR primers (Correa et al. 2009). A 10- μ l reaction mix containing 1 mM of both forward and reverse primers—1 μ l DNA template, 2 \times Absolute qPCR SYBR Green Fluorescein Mix (Thermo Scientific), and made up with dH₂O—was amplified in qPCR reactions (CFX96 real-time PCR detection system, Bio-Rad Laboratories, Inc.) using the FAM filter. Reaction conditions were an initial denaturing step of 95 °C for 10 min, followed by 50 PCR cycles of 95, 61, and 72 °C for 30 s each (Correa et al. 2009). A final high-resolution melting (HRM) step entailed a 55 to 95 °C temperature ramp, of 0.2 °C every 2 s. Fluorescence data were collected during each PCR annealing step, and each temperature step of the HRM melt cycle. Each DNA sample was run in duplicate for the clade D primer set, and positive (standard) and negative controls were included on every plate.

While RT-PCR primers lack the specificity to distinguish among *Durusdinium* species, *D. trenchii* (ITS2 type D1a, also known as D1–4) is (1) the species detected by DGGE and (2) understood to be the only representative of *Durusdinium* in the Caribbean Sea (Kennedy et al. 2015b). For these two reasons, we interpreted RT-PCR detection of *Durusdinium* as presence of *D. trenchii*.

DATA ANALYSIS.—Symbiodiniaceae communities and occurrences of low-abundance *D. trenchii* were compared within and between sites, and between years. An extended Fisher exact test of independence was used to determine shuffling between dominant symbiont species at each of the four locations, as well as for the region overall (Freeman and Halton 1951).

RESULTS

TEMPORAL STABILITY OF DOMINANT SYMBIONTS.—The symbiont communities sampled from 248 spatially independent *O. annularis* ramets were categorized using a combination of PCR-DGGE fingerprinting and sequencing. Every individual generated one bright farthest-migrating band on the gel, corresponding to the *B. minutum* symbiont (Fig. 2). In many lanes, additional bands were present. Sequencing revealed these either to be *B. minutum* heteroduplexes, or other *Breviolum* or *Cladocopium* species.

Profiles generated from Snapshot reef samples were homogenous and comprised one bright, low-molecular weight *B. minutum* band on a gel, and two or more slightly larger bands a short way above (Fig. 2). Sequencing revealed all three of these bands to be *B. minutum* heteroduplexes, in addition to confirming the dominant band as *B. minutum* (GenBank Accession no. AF333511). There was no observable difference in the dominant symbiont hosted by the Snapshot reef population across the four time points, with *B. minutum* dominating every sample tested (Fig. 3).

Propeller reef (P) displayed more variable DGGE profiles, although every sample had a dominant *B. minutum* band, and many showed heteroduplexes similar to those generated from Snapshot reef symbiont samples (Profile A). An additional slower-migrating band was present in several samples from 2006 (e.g., P03 and P06, Fig. 2, Profile A+). Although these bands appeared in a *Cladocopium* sp. C7 position (an *O. annularis* specialist), sequencing of this band revealed a different endosymbiont

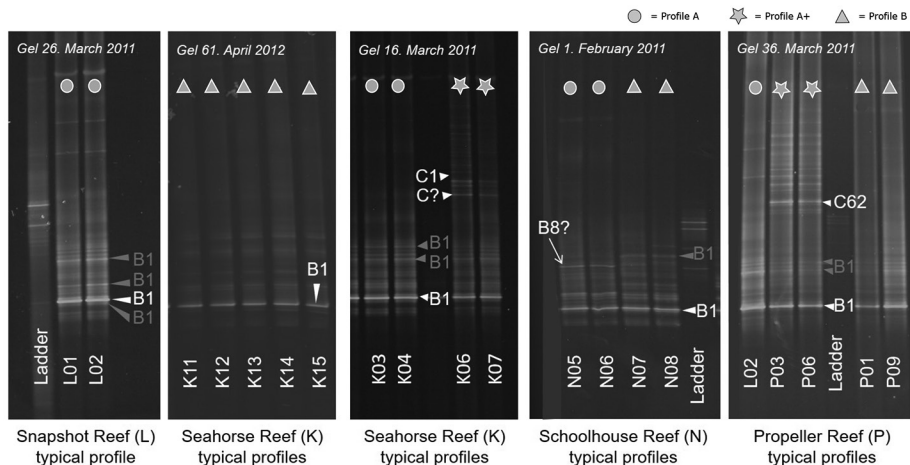


Figure 2. Denaturing gel gradient electrophoresis images revealing the most common banding patterns found at each site. *Breviolum minutum* (B1) was found in every single coral. *Cladocopium* ITS2 type C1 (C1) was also commonly dominant. Other species identified included a species similar to ITS2 type B8 (*Breviolum* sp.) and similar to ITS2 type C62 or maybe C7 (*Cladocopium* sp.)

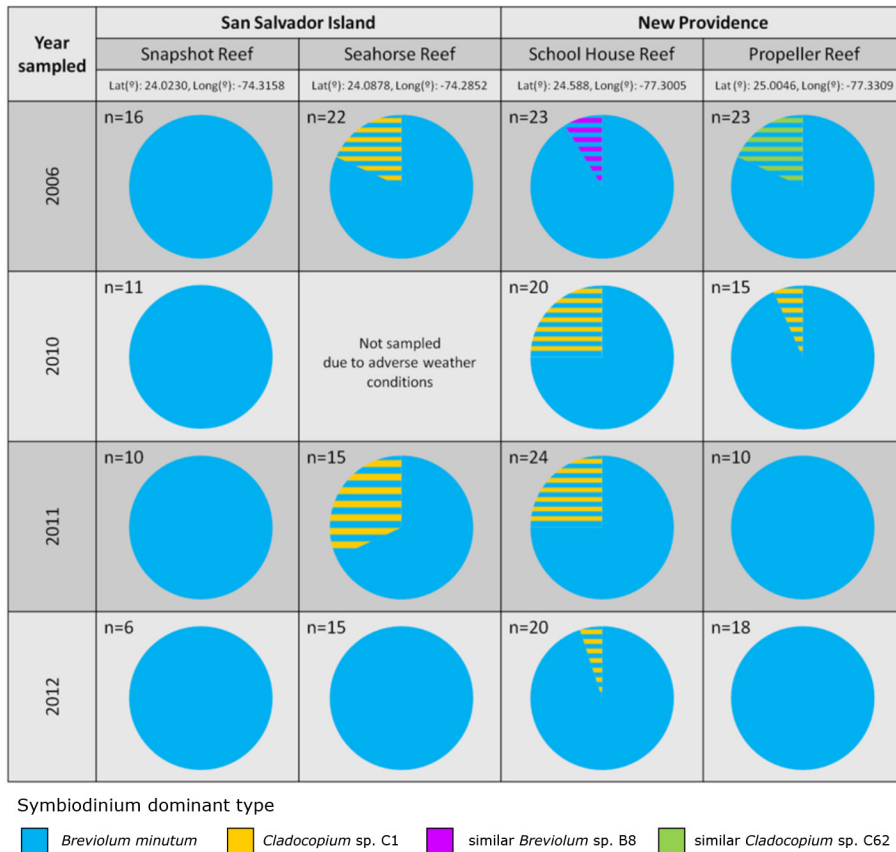


Figure 3. Pie charts representing dominant Symbiodiniaceae species harbored by *Orbicella annularis* ramets across four Bahamian reefs (Snapshot, Seahorse, School House, and Propeller reefs) at four time points: 2006, 2010, 2011, and 2012. Striped pie sections represent a pair of codominant types. Dominant types included *Breviolum minutum* (ITS2 type B1, GenBank Accession no. AF333511), *Cladocopium* spp. C1 (GenBank Accession no. AF333515), and two unknown types a *Breviolum* sp. similar to B8 (EMBL Accession no. = LR215821) and a *Cladocopium* sp. similar to C7a or C62 (EMBL Accession no. = LR215825).

type, which aligned best with *Cladocopium* sp. C62 in alignments with all known types (EMBL Accession no. LR215825 GeoSymbio; Franklin et al. 2012). This type has not been previously reported as inhabiting *O. annularis*. Propeller reef samples shared the faster migrating heteroduplexes present in Snapshot samples, but several samples also showed a slower-migrating band in the approximate *D. trenchii* position (GenBank Accession no. AF499802). Symbiont communities sampled in 2010 were all dominated by the *B. minutum* band, but again were quite varied, some communities displaying an uncommon lower band that was unable to be identified, although these were not dominant. The 2011 and 2012 samples appeared more uniform with little difference between symbiont communities hosted by each ramet.

School House reef (N) DGGE fingerprints revealed fairly uniform communities, with most samples (e.g., N07 and N08, Fig. 2) exhibiting profiles similar to Snapshot reef, with a band below *B. minutum* (identified as *Breviolum* sp. B10, GenBank

Accession no. AF499787), and a few samples showing slightly different banding, with a slower-migrating band around the *D. trenchii* position. However, re-running some School House reef samples adjacent on the same gel to some samples from Snapshot indicated the paler bands from School House were consistently in a different fingerprint position—slightly slower migrating than in the Snapshot profile. Sequencing of the dominant band and heteroduplexes revealed all to be *B. minutum* or heteroduplexes. The unusual band in N05 and N06 also had a different sequence (Fig. 2, EMBL Accession no. LR215821); however, the method was unable to confidently identify this symbiont—a BLAST search revealed *Breviolum* sp. B8 to be the closest match. The 2010, 2011, and 2012 samples all generated a mix of profiles, with some co-dominant for *B. minutum* and *Cladocopium* sp. C1 (GenBank Accession no. AF333515), but most being exclusively dominant for *B. minutum*. Seahorse reef (K) showed relatively simple profiles dominated by a single band in the *B. minutum* position. A much slower-migrating band (*Cladocopium* sp. C1) was often co-dominant with *B. minutum*.

An extended Fisher exact test confirmed that the relative proportion of dominant *Cladocopium* and *Breviolum* clades observed remained independent of the sampling time point ($P = 0.088$, Table 1). This result was also confirmed at site level, except for at Seahorse reef, where a significant change in the proportion of *Cladocopium* symbionts hosted between 2011 and 2012 meant that community composition was not independent of time ($P = 0.042$, Table 1). Overall, these results suggest temporal stability in the dominant types hosted by *O. annularis* across The Bahamas, and at all reef sites included in this study, with the exception of Seahorse Reef.

TEMPORAL STABILITY OF CRYPTIC *DURUSDINIUM TRENCHII*.—*Durusdinium trenchii*, or D1a, was detected in *O. annularis* colonies at all four sites, and also at all four time points (Fig. 4). Where *D. trenchii* was present at a site, it was harbored by 5%–42% of colonies. Over time, there appeared to be a decline in the number of colonies hosting low abundances of *D. trenchii*, from an average 23% of colonies (across all sites) in 2006, to 12% in 2010, to 9% in 2011, and just 1% in 2012. By 2012, *D. trenchii* was only detected at one site (School House reef) and in just one sample (Fig. 4).

Table 1. Results of Fisher's exact test comparing temporal changes in the dominant symbiont hosted at each location, as determined by denaturing gel gradient electrophoresis analysis, demonstrates that relative proportions of dominant symbionts did not change from year to year at the 1% significance level (significant differences highlighted with an asterisk). Bold indicates total for all sites. ^a Seahorse reef was excluded from The Bahamas total calculation, as data were missing from 2010.

Location	df	<i>P</i>
Snapshot reef	0	1.000
Seahorse reef	2	0.042*
School House reef	6	0.152
Propeller reef	6	0.215
The Bahamas (total)^a	3	0.088

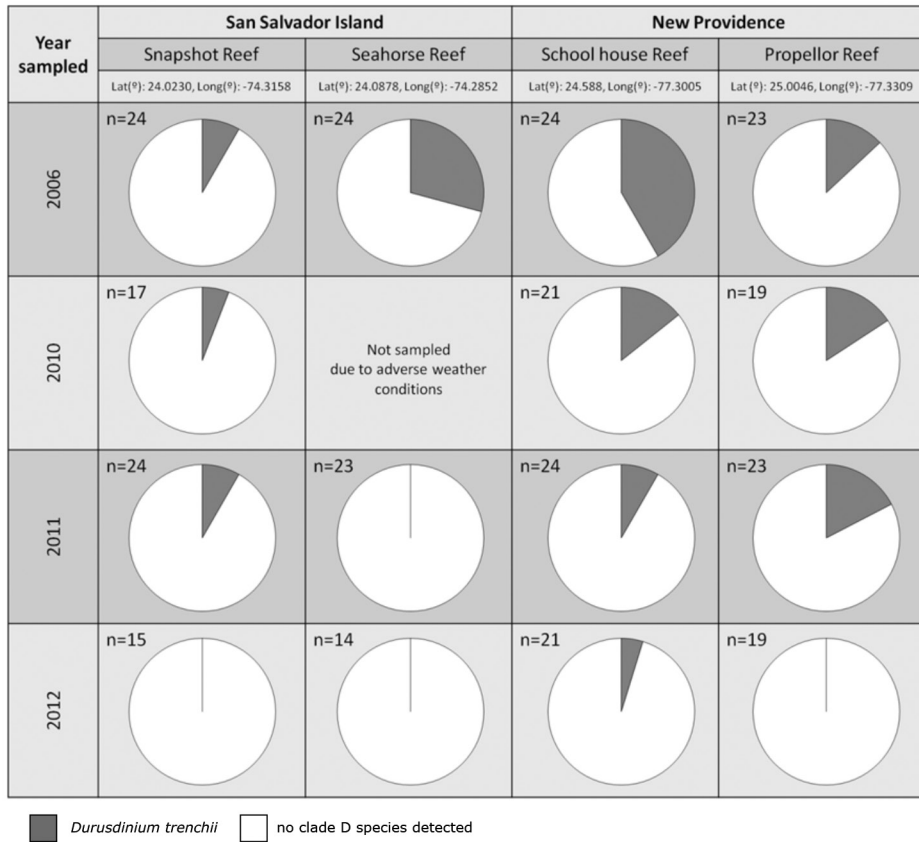


Figure 4. Pie charts displaying changes in the relative number of *Orbicella annularis* ramets found to host low abundances of *Durussdinium trenchii*, or D1a, at four different sites in The Bahamas (Snapshot, Seahorse, School House, and Propeller reefs), at four time points (June of 2006, 2010, 2011, 2012).

Extended Fisher exact tests suggest temporal differences in the number of colonies hosting *D. trenchii* at two of the sites—Seahorse reef and School House reef—but not at Snapshot or Propeller reefs (Table 2).

DISCUSSION

The DGGE data show broad temporal stability of dominant symbionts in *O. annularis* in The Bahamas over a 6-yr time period, suggesting that spatial patterns in symbiont biogeography are likely to be fairly robust over time periods of 5–10 yrs. This stability appears to be a feature of not just *O. annularis*, but other Caribbean species (Thornhill et al. 2006a, Warner et al. 2006). The finer scale RT-PCR technique revealed interannual variability in background abundances of cryptic species *D. trenchii*. Together, these results suggest that, while it might take an extreme environmental perturbation to trigger a shift in the dominant symbiont or symbionts, symbiont species within the community are likely to be experiencing

Table 2. Results of Fisher's exact test comparing temporal changes in *Durudinium trenchii* hosted by *Orbicella annularis* ramets at four Bahamian locations, as determined by high resolution melt analysis, showing that the relative proportions of ramets hosting *D. trenchii* were dependent on sampling year. Seahorse and School House reef symbiont communities were different across years at the 1% level, with fewer ramets hosting *D. trenchii* in 2012 compared to in 2006 (significant differences highlighted with an asterisk). Bold indicates total for all Bahamas sites.

Location	df	<i>P</i>
Snapshot reef	3	0.859
Seahorse reef	2	0.002*
School House reef	3	0.006*
Propeller reef	3	0.306
The Bahamas (total)	3	<0.001*

continual shuffling in their relative abundances as they jostle in response to seasonal or environmental changes.

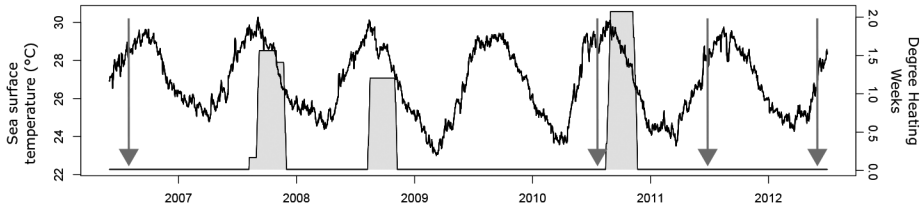
TEMPORAL STABILITY OF DOMINANT SYMBIONT TYPES.—At all four study sites, at all sampling time points, ITS2-type B1 (*B. minutum*) was found to be the dominant species in every community, and was hosted by 100% of colonies. Snapshot reef showed a stable community of *B. minutum*, in all samples, over the 6-year sampling period. At Propeller, Seahorse, and School House reefs, *B. minutum* was shown to share dominance with *Cladocopium* sp. C1 in a proportion of colonies, while in 2006, *B. minutum* from four colonies at Propeller and two colonies at School House reef was co-dominant with *Cladocopium* and *Breviolum* symbiont types that could not be confidently identified (possibly C62 and B8, respectively). However, annual variation in the absolute number of ramets hosting a dominant pair of symbionts (as opposed to exclusively *B. minutum*) were not deemed significant by Fisher's exact tests ($P > 0.01$). These findings imply substantial temporal stability over a 6-yr time period.

It is difficult to explain the difference between Seahorse reef, which had more ramets hosting mixed symbiont assemblages, and the other sites; this offshore reef site was considerably more exposed than the others, and differences between inner and outer reefs (generally driven by water quality factors) are consistently identified as important in driving symbiont community partitioning (Garren et al. 2006, LaJeunesse et al. 2010, Cooper et al. 2011).

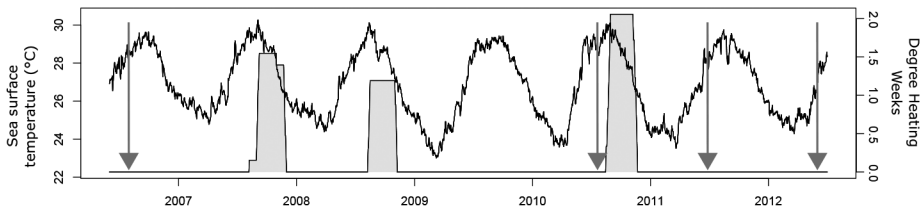
Overall, the result appears to be consistent with the other studies that have demonstrated broad temporal stability of *O. annularis* symbiont communities—in the absence of severe thermal stress events—over comparable time periods (e.g., 2 yrs in Warner et al. 2006; 5 yrs in Thornhill et al. 2006b). Researchers sampling 12 tagged *O. annularis* colonies in The Bahamas 15 times between August 2000 and August 2004 similarly found all colonies were dominated by *B. minutum* throughout the duration of sampling, although a few deeper colonies (12 m) had several instances of mixed ITS2 B1 and C12 (Thornhill et al. 2006b). In another study, the same researchers found that none of the genetic variation within the *B. minutum* populations was attributable to sampling time points from *O. annularis* [or *Orbicella faveolata* (Ellis and Solander, 1786)] across The Bahamas (or Florida) (Thornhill et al. 2009), with reef location being more important in explaining diversity (indeed, in *O. annularis* all symbiont genotypes were site specific).

However, two thermal stress events, one in November 2005, just 8 mo prior to the first sampling point, and a second in October 2010, between the second and third

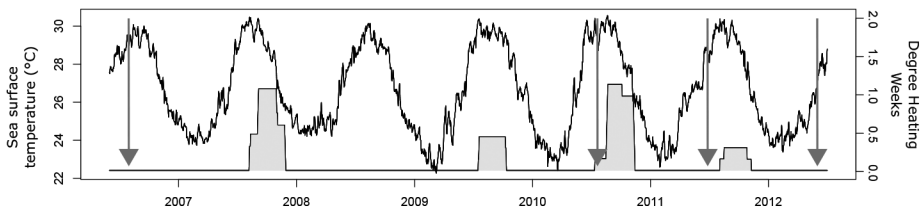
Snapshot Reef



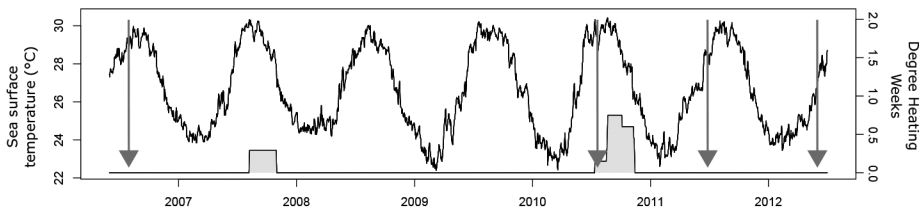
Seahorse Reef



Schoolhouse Reef



Propeller Reef



Sea surface temperature (°C)
 Degree heating weeks (monthly maximum)
 Corals sampled

Figure 5. Maps showing sea surface temperature (dark line) and Degree Heating Weeks (grey bars), a measure of thermal stress, for the four Bahamian sites during the study period (2006–2012). Data derived from NOAA Coral Reef Watch (2013) satellite products.

time points, occurred in the region (Fig. 5). These sea surface temperature (SST) anomalies might have been expected to influence symbiont community composition, especially if bleaching was triggered in the colonies studied here. Bleaching events near the study sites were reported by local scientists and dive operators, although no quantitative estimates exist for these periods. On San Salvador during November 2005, the Bahamian Reef Survey team (Earthwatch Institute) noted that “bleaching

was evident primarily on *Agaricia* sp., *Favia fragum*, and *Porites* sp. ... limited bleaching of *Orbicella annularis* was also observed; however, many coral heads of this species displayed significant blanching” (Rollino 2005). The bleaching reported in 2005 does not appear to be as severe as the 1998 event, where 60% of all *O. annularis* bleached on San Salvador Island (Peckol et al. 2003, McGrath and Smith 2005). The principal scientist from the Earthwatch team, John Rollino, compared his observations to the milder 1995/1996 bleaching event, where just 2% of *O. annularis* colonies bleached (McGrath and Smith 2005). Bleaching was described in San Salvador by the same team in December 2010, again noting *A. agaricites* colonies bleaching >75% of their surface, and mortality in their 15-mo old *Acropora palmata* (Lamarck, 1816) transplants, although it was noted that *Orbicella* sp. exhibited “little, if any, ill effects” (Rollino 2010). Meanwhile, bleaching was also reported in October 2010 by Stuart Cove Dive Operators at sites around the south east of New Providence (School House and Propeller reefs). “All sites exhibited some bleaching” (Rollino 2010), although more detailed information on the bleaching severity and impact on different species was not provided.

Thermal stress events clearly affected the reefs of San Salvador and New Providence over the period of our study. However, the lack of a severe bleaching response in *O. annularis* may explain why dominant symbionts hosted were maintained throughout the study period. Whether comparable temporal stability in symbiont dominance would have been maintained throughout a more severe bleaching event—such as observed in 1998—is unknown. *Breviolum minutum* has clearly maintained its dominance over several warmer-than-usual periods (Fig. 5) and demonstrated a high degree of temporal stability in the holobiont of *O. annularis* in this region.

TEMPORAL VARIABILITY OF CRYPTIC *DURUSDINIUM TRENCHII*.—The second part of the present study focused on a thermally tolerant symbiont known to be present in relatively low densities in *O. annularis* colonies (Kennedy et al. 2015a). *Durusdinium trenchii*, or D1a, was detected in a proportion of colonies at all sites (at least one time point), but occurrence diminished over the study, with just one colony (out of 69 sampled in 2012) observed to contain *D. trenchii* in 2012, compared to 22 colonies (out of 95) sampled in 2006. At Seahorse and School House reefs, observed differences between sampling time points were shown to be significant by Fisher’s exact tests ($P < 0.01$).

This result is consistent with previous studies that have reported an eventual post-bleaching reversion to original symbiont community types, which involves a reduction in the presence of *D. trenchii* (thought to incur trade-offs in terms of calcification efficiency) following increases in density during (or in the buildup to) bleaching (Thornhill et al. 2006b, LaJeunesse et al. 2009). It has been suggested that *D. trenchii* may naturally persist at trace background levels within symbiont communities, and that stress events, such as elevated SSTs, may trigger a shift in relative abundance to detectable levels (Kennedy et al. 2015a). It is possible that the “blanching” observed by John Rollino in Bahamian *O. annularis* in November 2005 facilitated shuffling of low *D. trenchii* abundances to detectable levels 7 mo later, as observed in our 2006 data. Other studies show high amounts of *D. trenchii* prior to, during, and immediately after bleaching (Thornhill et al. 2006b, LaJeunesse et al. 2009). In a study of symbiont assemblages in Barbados, levels of *D. trenchii* remained high for at least 7 mo following a bleaching event in October 2005, and were still

comparable with bleaching levels in April/May 2006, only dropping off after 24 mo (LaJeunesse et al. 2009). In a Florida study, *D. trenchii* was detected in the tops of *O. annularis* colonies prior to the 1998 bleaching event, and remained detectable (by DGGE) in a community of six coral colonies until May 2002, after which it was not again detected (the study ended in August 2004) (Thornhill et al. 2006b). Without sampling prior to 2005, it cannot be known whether these levels (23% of colonies) are typical for Bahamian *O. annularis*, although evidence suggests at least 30% of colonies across the Caribbean region contain cryptic *D. trenchii* (Kennedy et al. 2015a), and 28% of healthy *O. annularis* colonies contained cryptic *D. trenchii* in Barbados (LaJeunesse et al. 2009).

Lack of survey data from 2007 to 2009 creates uncertainty as to whether prevalence of *D. trenchii* declined, or was maintained, between our first two sampling time points. If thermal stress is important in influencing *D. trenchii*, the 2008 accumulated Degree Heating Weeks (DHWs) (Fig. 5) may have affected the number of colonies hosting *D. trenchii*, although no bleaching reports were reported for our sites in 2008. The colonies from 2010 were sampled 6 mo prior to reported reef bleaching, although incidences of low abundance D1a have been shown to accumulate in *O. annularis* in the buildup to bleaching events (LaJeunesse et al. 2009). The 12% prevalence of *D. trenchii* we recorded in 2010 may be a legacy from the 2005 (or possible 2008) thermal stress events, or as an immediate response to the elevated temperatures observed in June 2010 (Fig. 5). The latter seems more likely, as if the declining trends we observe are genuine, then *D. trenchii* densities are clearly able to drop to undetectable levels within the space of a year (e.g., 2011 to 2012) if environmental conditions are favorable.

In 2011, *D. trenchii* was detected at three of the four sites studied: again, 6 mo after a reported bleaching event (albeit one in which bleaching was not specifically reported in *O. annularis*). *Durussdinium trenchii* was barely detected in 2012; if abundances are linked to thermal stress events, this may be due to more stable temperature conditions enabling other symbionts to gradually re-establish full dominance, thereby supporting previous studies suggesting post-bleaching reversion in *O. annularis* (Thornhill et al. 2006b).

Two of our study sites, Snapshot and Propeller reef, did not show significant changes in the number of colonies hosting *D. trenchii*, while Seahorse and School House reef clearly did. Propeller reef—situated relatively near to a warm water outflow—may have maintained a high level of background *D. trenchii* for this reason. It is impossible to reliably attribute the decline in the number of ramets hosting detectable *D. trenchii* to thermal stress events with this data set; however, it seems likely that thermal stress may be providing a mechanism by which low level densities of different symbiont taxa are in a continual state of flux. *Orbicella annularis* is documented as being one of the first species to bleach and has been shown to be more susceptible to water temperature increases than other corals (Fitt et al. 2001). Perhaps a fluctuating presence of *D. trenchii* reported in the present study may explain why, in 2005 and 2010, *O. annularis* in The Bahamas appeared to avoid bleaching.

In summary, we revealed temporal variability in the presence of a low-abundance, but potentially ecologically-important symbiont, in key reef-building corals in The Bahamas, despite broader apparent stability in the dominant community members over a 6-yr period. While the results support other studies that show long-term temporal stability of associations between *O. annularis* and *B. minutum*, it also highlights

the importance of the nuances in symbiont community stability over time, and the importance of detection limits.

With coral reefs facing an unprecedented global crisis (Hughes et al. 2017), understanding these nuances in the coral microbiome will be important in interpreting bleaching responses and determining outcomes for reefs. Underlying mechanisms, such as shuffling of symbiont communities at a micro-scale, have consequences for individual coral colonies, and for *O. annularis*—an abundant species and dominant reef builder—these small effects may contribute to a coral reefs wider ability to resist or rebound from thermal stress in the face of a rapidly changing climate (Guest et al. 2018).

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ETHICS

The project was assessed and approved by the Department of Biosciences Ethics Committee, University of Exeter, UK. The necessary permits for collection and export of coral samples were provided by the Department of Fisheries, Nassau, The Bahamas. CITES import permits and Animal Health Licences were provided by the Department for the Environment, Food and Rural Affairs, Bristol, UK.

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